by a yeast promoter and by the yeast termination DNA sequence. Plasmid arrangement is protective of the whole DNA sequence.

In the current invention, the *P. pastoris* is transformed with recombinant plasmid, such as pPICZαLH and zeocin-resistant transformants are isolated.

A schematic representation of expression cassettes which are unique to this invention is shown in Figure 1 of the specification. The recombinant plasmid, pPICZαLH, contains a bacterial origin of replication (COIEI), Zeocin-resistance gene (Zeo^R) for selection of both *E. coli* and yeast transformants, and the expression cassettes of antibody light and heavy chain DNAs exemplarized by the anti-dioxin antibody DNAs.

The final construct is assembled by replacement-ligation of the *MluI-Bam*HI fragment (for dioxin) of the recombinant plasmid construct containing the light chain expression cassette, and the *MluI-Bam*HI fragment from the heavy chain expression cassette construct.

As seen in Figure 1, the expression cassettes of the light-chain (L-chain) and heavy-chain (H-chain) DNAs are each fused to the yeast α-factor signal sequence (SS), under the control of the yeast promoter (AOX1-P) at 5'-terminal. A yeast transcription termination sequence (TT) marks the 3' end of each expression cassette. The expression cassettes are thus special entities expressed as such and are protective of the antibody intactness. They are not expressing, and do not permit expression of, other entities such as individual DNA fragments.

Results obtained with using the expression cassettes as illustrated in the specification for dioxin, confirm the integrity of expression cassettes and the junction sequences of antibody structural and spatial arrangement by DNA sequence analysis.

PCR analysis of *P. pastoris* transformants prepared according to the invention using primers specific for the antibody genes or for the AOXI 5' and 3' termini shows intact full-length light- and heavy-chain gene expression cassettes integrated in genomic DNA of the transformants. Control transformants harboring vector alone yield no amplification products in PCR with Ab gene primers. DNA sequence analysis of the PCR products from recombinants is used to confirm that the primary structure and spatial orientation of the target sequences is preserved (Specification, page 19, lines 21-29).

The antibody production by recombinant *P. pastoris* clone is easily induced. Induction of recombinant antibody expression is typically performed as follows. A *P. pastoris* transformant is cultured for two days at 30°C. The yeast cells are collected by centrifugation and transferred to the induction medium. Beginning on the second day of growth, methanol is added daily to induce the AOX1 promoter-driven production of recombinant antibody.

The method involves making a nitrocellulose membrane-replica of recombinants on an agar plate with induction medium and probing it with AP-goat anti-mouse IgG.

Transcripts detected in the Northern blot are specific and correspond to the sizes expected for both light- and heavy chains.

The intactness of the inserts and the accuracy of the junction sequences are easily confirmed using PCR procedure by nucleotide sequence analysis.

Antibody levels in cell lysates is approximately 10% of the total product, as assessed by Western blotting, and are consistently lower than those found in culture medium. This demonstrates that a major portion, approximately 90% of the antibody produced by recombinant *P. pastoris*, according to the invention, is secreted into the supernatant in an amount from about 10-36 mg/l in about 12 hours to 5 days. These results show that *P. pastoris* secretes intact antibody very efficiently. These results are unexpected, surprising and very advantageous for rapid preparation of large quantities of a very specific antibody.

Studies performed to determine the kinetics of monoclonal antibody have shown that synthesis and secretion of antibody are optimal between 72 and 108 hours. Using the current method, anti-hapten antibody was detectable in culture medium between 12 hours and 120 hours of induction, with highest levels of about 10 to 36 mg/l detected between 72 and 108 hours.

Although these levels are lower than those reported for other recombinant proteins or for antibody fragments (200 mg/l), they are the highest and set the highest range ever obtained for any intact antibody, which is a more complex multimeric glycoprotein, than the levels previously reported. These levels can be further augmented by using fermentation approaches.

This invention thus provides a method of general utility for production of large quantities of any antigen-specific antibody using modified yeast organism. Using the method of invention, the large quantity of antigen specific and defined monoclonal antibody is produced without the necessity of immunizing and recovering and purifying antibodies and/or other lengthy procedures. The method is practical, economical, easy, safe and fast and in about three days, the monoclonal antibody is produced by the transformed yeast if the vector and expression vehicles for transformation are available or are prepared according to the invention.

The above described findings demonstrate the suitability of *P. pastoris* expression system for both small and large-scale production of functional, antigen-specific and structurally and spatially intact antibodies. The recombinant antibodies produced by the method of the invention are useful, for example, for immunodiagnostic and immunotherapeutic purposes. Since recombinant proteins produced in *P. pastoris* lack terminal α-1,3-glycan linkages responsible for hyper-immunogenicity, the antibodies produced in *P. pastoris* are particularly suitable for therapeutic applications.

Rejections under 35 U.S.C. 103

Examiner rejects claims 22-24 and 27-32 under 35 U.S.C. 103(a) as being unpatentable over Horwitz et al (Proc. Natl. Acad. Sci. USA 85:8678-8682, 1988) and further in view of Cregg et al (Developments in Industrial Microbiology 29:33-41 1988) and The Invitrogen 1997 Catalog (published 1/97, Yeast expression pages 14-

19 and Master Catalog Amendment Notice for pPICZ vectors from 4/15/96) and Sambrook et al (Molecular Cloning A Laboratory Manual Second Edition pages 1.85, 12.16-12.20, and 13.42-13.44, 1989) is maintained for reasons of record.

Applicants disagree. Horwitz reference describes secretion of functional antibody and the Fab fragment from yeast cells. Cregg teaches production of foreign protein of noncomplex nature and only in general terms. Invitrogen describes a method for production of large quantities of simple proteins. Sambrook describes general methods used in molecular technology.

According to Horwitz, to construct the functional antibody, cDNA copies of the chimeric light chain and heavy chain genes were inserted into separate vectors for each chain (see Figure 2A). The vector pING1441 comprising the complete 2-μm plasmid, the chimeric light chain gene V and C_K regions fused to the PGK promoter P invertase signal sequence S and PGK transcription termination and polyadenylation signal T. The vector pING1442 for heavy chain contains the yeast origin replication (oriY) and a cis-acting stabilization sequence Rep3 from yeast 2-μm plasmid, the chimeric heavy chain gene V-region and C region domains C_{H1}, C_{H2} and C_{H3} fused to PGK promoter, invertase signal sequence and PGK transcription termination and polyadenylation signals.

The used plasmids are entirely different entities from each other which are unconnected and structurally unrelated to each other.

A cloning strategy of Horwitz consists of fusing cDNA coding for the mouse V regions at the immunoglobulin joining J region to cDNA coding for human IgG1 C regions.

While the method of Horwitz would seem to produce antibody containing both heavy and light chains using different vectors and methods, the production is extremely inefficient as only about 100 ng (nanograms) per ml is produced in 3 days which corresponds to about 100 micrograms per liter, compared to the current method producing, that is about 10-36 milligrams per liter during the same time.

Moreover, as Examiner admits, Horwitz does not teach a recombinant host *P. pastoris* transformed with a vector for expression, the AOX1-P promoter, the pPICZa vector, replacement of the yeast chromosomal AOX1 with the AOX1-antibody DNA by homologous recombination, or selection on zeocin media or screening by colony-immunoblotting, restriction analysis or nucleotide sequence analysis. These deficiencies, however, according to the Examiner, are made up for in the teachings of Cregg et al, the Invitrogen 1997 Catalog, and Sambrook et al.

Applicants do not think so. As described in the current specification, production of antibodies is much more complicated that production of recombinant proteins as described by Cregg. A synthesis of antibody, which is a complex multimeric glycoprotein present challenging problem. Cregg teaches that yeast can be used for production of foreign proteins but does not describe production of antibodies. Only simple proteins, such as enzymes β-

galactosidase and alcohol oxidase. Nowhere in Cregg is there any suggestion that *P. pastoris* would be suitable for production of complex molecules such as antibodies. As a matter of fact, there is nowhere in Cregg paper any coherent description of the methodology which the authors were using and it would be very difficult, if not impossible to reproduce the method even for the simple proteins which it is suppose to produce.

The fact remains that although both Horwitz and Cregg references were available to persons skilled in the art since 1988 and although clearly there was, and still is, a need to produce complex multimeric proteins in large quantities to make them therapeutically significant surpassing those produced in animals, in a shorter amount of time than needed for production of antibodies by animals, a method for producing such intact antibodies in described quantities is still not available. That alone shows that the invention is not obvious from Horwitz in view of Cregg.

Examiner additionally rejects claims 22-24 and 27-37 on the basis of combination of Horwitz, Cregg with Invitrogen Catalog. Examiner argues that Invitrogen discloses production of proteins in gram per liter quantities. That may be good and well, but Applicants invention is not directed to production of simple proteins such as those listed in Invitrogen Catalog, for example, enzymes, proteases and protease inhibitors, membrane proteins, antigens and regulatory proteins. Applicants are producing complex molecules which are entire and intact monoclonal antibodies.

Examiner would like to overcome a clear lack of nonobviousness by adding reference Invitrogen which makes the *Pichia pastoris* pPICZα vector available for cloning and selection. But again, until late 1998, no person skilled in the art came up with such, according to the Examiner, "obvious" solution of the truly serious problem which still remains.

Invitrogen can be used to express antibody fragments, probably in the rate of production which Examiner is citing. However, it is generally known, accepted and described in quite a few publication that although prokaryots, eukaryots and a unicellular eukaryots namely, such as the baker's yeast, Saccharomyces cerevisiae, and the methylotrophic yeast, *Pichia pastoris*, are able to produce intracellular and extracellular proteins, including antibody fragments, these organisms are not able to express structurally and spatially oriented complex proteins, such as intact antibodies, as described in, for example, in Nature Biotechnology, 16:773 (1998), J. Biochem., 121:831 (1997), BioTechnology, 13:255 (1995), PNAS (USA), 85:8676 (1998).

In order for *P. pastoris* to produce such intact complex antibodies in quantities as claimed, the method for their expression needed to have been developed, modifications and sequence of the step of claim 1 must have been invented, tested and their efficacy determined.

The problem with expression of antibodies rests with their structural arrangement. Antibodies contain several fragments, which individually could possibly be expressed by Invitrogen

Catalog method as individual fragments, but cannot be expressed as the whole intact protein comprising these fragments without modifications of the Invitrogen method by invention steps.

Antibodies (Monoclonal Antibodies: Principles and Practice, pp. 7-10, Ed. J. W. Goding, Academic Prep, Inc., London (1983)) (copy was previously submitted) are complex symmetrical molecules made up of two identical glycosylated heavy chains of molecular weight typically between 50,000-75,000 and two identical nonglycosylated light chain of molecular weight around approximately 25,000. The heavy chains are joined by disulfide bonds to each other and each light chain is joined by a disulfide bond to one heavy chain.

Each chain is further made up of a series of homology units of approximately 110 amino acids. Each homology unit contains one intra-chain disulfide bond between cysteine residues situated about 20 amino acids from each end.

Each homology unit is folded into a domain, which is a compact, globular structure containing large amounts of β-plated sheets. Antibodies are thus structurally and spatially complex molecules built of the different interconnected domains. These domains are encoded by different genes, as seen in Figure C of Immune System, Color Atlas of Genetics, E. Passarge, Thieme Medical Publishers, Inc., New York (1995). Light chains, which are lower molecular weight proteins comprising one variable and one constant region, are encoded for by different genes than the heavy chains, which are proteins comprising one variable, one hinge and three

constant regions. Each region is encoded for by different V_H (variable), CH1, H, CH2 and CH3 genes for heavy chains.

Proteins which are conveniently expressed with Invitrogen are simple proteins encoded for by one gene and not a complex proteins encoded by at least two genes, each encoding one specific region of the antibody, which regions additionally need to be spatially and structurally interconnected with each other by way of disulfide bridges and other bonds.

The above is just a brief description of complexity of the antibody molecule. Monoclonal antibodies additionally contain other distinguishing features, however, the above description is deemed to be sufficient for the purpose of illustrating the complexity of antibody molecule. No such complexity exists in a regular protein which can be easily expressed by the Invitrogen method and kits.

Applicants maintain that the Invitrogen kit and method does not produce the complete intact and functional antibody as described above and that their (Applicants') method developed just for this purpose, does. Only if such a structure can be reproduced in its entirety, the antibody of the invention, which is complete, intact and functional antibody, is obtained. It is respectfully submitted that Invitrogen methods and kits do not produce this complex structure and that Invitrogen combination with Horwitz and Cregg would not lead to production of large quantities of intact antibodies.

It is respectfully submitted that claims 22-32 are not obvious and requested that Examiner reconsider his rejection on the basis of these remarks.

The rejection of claims 33-35 under 35 U.S.C. 103(a) as being unpatentable over Horwitz et al and further in view of The 1997 Invitrogen Catalog is maintained for reasons of record.

Claims 33-35 are cancelled and rejection is therefore moot.

Examiner further rejects claims *25-26* under 35 U.S.C. 103(a) as being unpatentable over Horwitz et al (PNAS 85:8678-8682, 1988) as applied to claims 22-24 above, and further in view of Cregg et al (Developments in Industrial Microbiology 29:33-41, 1988) and The Invitrogen 1997 Catalog published 1/97, Yeast expression pages 14-19 and Master Catalog Amendment Notice for pPICZ vectors from 4/15/96), Sambrook et al (Molecular Cloning, A Laboratory Manual Second Edition pages 1.85, 12.16-12.20, and 13.42-13.44, 1989) and Vanderlaan et al (U.S. Patent 5,429,925, issued 7/4/95) is maintained for reasons of record.

Applicants disagree. The fact remains that no combination of references cited by the Examiner would produce the dioxin-specific antibody in quantities, as disclosed in the specification and claimed in claims 22, 25 and 26, as amended. The Examiner combines five references which, individually or in combination, that is, one in view of another, do not achieve the result of the current invention.

The current method, encompassed in the currently amended claims, produces large amounts of the whole intact antibodies in

amounts from about 10 milligrams to about 36 milligrams in from 12 to 108 hours (half a day to about 5 days). No method described before ever achieved the same results.

The closest method Examiner can come up with is the method of Horwitz, which produces 100 micrograms of antibodies in 3 days, that is, 100-360 times less than the current method and it takes six (6) times as long as the shortest time (12 hours) of the current method when at least 10 milligram of intact entire antibody is produced.

Examiner argues, without providing any evidence, that the current method is obvious because by combining Horwitz with Cregg, with the Invitrogen Catalog and with general methods described in Sambrook et al., Molecular Cloning, A Laboratory Manual, and with Vanderlaan, one would expect, or have reasonable expectation, that the resulting combination could have produced a high level of intact antibody in a shorter amount of time.

To that, Applicants respond that even if, and Applicants do not agree with this reasoning, but even if the combination of all four cited references would produce a higher level of intact antibodies in a shorter amount of time, would it be really reasonable to expect to produce more than 100 to 360 times the amount as described previously by Horwitz. Such a large increase in a yield is not achieved simply by combining five unrelated references without extensive research, testing, retesting and method modifications, as detailed in the claims.

Applicants do not understand how "reasonable expectation" could include 100-360 times higher yield. Reasonable expectation may include 1-10 times higher yield but to reasonably expect 100-360 times higher yield by arbitrarily combining five references is unreasonable.

Applicants maintain that it is unreasonable for Examiner to insist that the combination of Horwitz, who is able to produce only a very small amount of the antibody in three days, using a totally different method from the current invention, with Cregg, et al., who at most teaches that a yeast is easily scaled up from shake-flask to large volume, high density cultures and that the yeast can be used for production of foreign proteins would derive the current invention. Does Cregg teach such scale-up, does he show large production of proteins, antibodies or any specific protein? Applicants cannot find any such teachings. All he does is describe the production of foreign proteins of noncomplex nature in general terms. There is no experimental section in this paper and no results or yields of any of his experiments are given. Applicants cannot understand how would this paper lead to production of large amounts of antibodies which are rather complex multimeric glycoproteins and which are, even under the best of circumstance, much more difficult to produce and particularly reproduce in their entirety than the simple proteins.

Examiner produces lengthy justifications for his rejections of this application. Applicants maintain that if the invention would be truly obvious, such dissertations would not be necessary. To

argue that an increase in yield of the antibody production by more than 100-360 times is obvious in view of unproven combination of four unrelated references is erroneous and unsupportable.

The rejection should be withdrawn and all remaining claims should be passed to issue.

SUMMARY

In summary, claims 22 and 25-32 are pending, claim 22 is amended to introduce further limitations taking into consideration Examiner's rejections. Applicants provided arguments showing that the invention is not obvious from the prior art. It is believed that with this amendment, all claims are in condition for allowance. Notice of Allowance is respectfully requested.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES

22.(Amended) A method for a production of about 10-36 mg/l of a recombinant antigen-specific entire intact monoclonal antibodies having preserved a primary structure, in about 12 hours to about 108 hours, said method comprising steps:

(a) isolating, chemically synthesizing or amplifying with polymerase chain reaction (PCR) a cDNA, mRNA or genomic DNA encoding a light or heavy chain of the antigen-specific antibodies and assembling the antibodies cDNA encoding said light and heavy chains of said antibodies into two separate expression cassettes, one encoding DNA for the light chain and the second encoding DNA for the heavy chain, each cassette further comprising a flanking signal DNA sequence preceded by a yeast promoter at 5' terminus and by the yeast transcription termination DNA sequence of the 3'-terminus,

wherein the antibody cDNA is assembled into the expression cassettes by subcloning the antibody light and heavy chain cDNA in tandem as *Eco*RI-*Bgl*II/*Bsm*BI fragments flanked by a DNA encoding the *P. pastoris* signal sequence, preceded by a *P. pastoris* promoter at the 5'-terminus and by a *P. pastoris* yeast transcription termination DNA sequence at the 3'-terminus, and

wherein the signal sequence is a yeast α-factor and wherein the promoter is an alcohol oxidase AOX1-P;

(b) preparing a recombinant *Pichia pastoris* (*P. pastoris*) yeast expression vector pPICZα by restriction digestion

with EcoRI and BamHI;

(c) constructing a recombinant *P. pastoris* yeast expression plasmid containing the expression cassettes of step (a);

(d) cloning the expression cassettes of step (c) into the *P. pastoris* expression vector pPICZα to generate recombinant plasmid pPICZαLH comprising expression cassettes for the light and heavy chains;

(e) transforming *Saccharomyces cerevisiae* with the recombinant plasmid by placing said expression cassettes of step (d) under the control of the AOX1 promoter fused to the DNA encoding the *Saccharomyces cerevisiae* α-mating factor signal;

(f) amplifying and isolating the recombinant plasmid;

(g) transforming *P. pastoris* spheroblasts with *Bgl*II linearized, *Not*I linearized, *Sac*I linearized, *Sal*I linearized or *Stu*I-linearized recombinant plasmid replacing the yeast chromosomal AOX1 DNA sequence with AOX1-antibody DNA sequence containing expression cassettes of the recombinant plasmid of step (d);

(h) selectively growing the recombinants;

(i) screening yeast transformation colonies for a recombinant antibody expression;

(j) analyzing putative positive yeast clones for chromosomal integrates of the expression cassettes of heavy and light chain cDNAs;

(k) confirming the integrity of the DNA insert;

(l) inducing the recombinant antibody expression;

(m) confirming the intactness of the expression

cassettes inserts with PCR and Northern blot analysis;

(n) detecting the presence of the recombinant antibody by Western blot;

(o) testing the recombinant antibody for specific antigen-antibody binding, and

(p) harvesting the antigen-specific antibody produced in steps (a) - (o);

wherein said antibody is produced in quantity of 10-36 mg/l in about 12 to about 108 hours.

25. (Amended) The method of claim [24] 22 wherein the antigen is dioxin.

27. (Amended) The method of claim 22 [24] wherein the replacement of the yeast chromosomal AOX1 with AOX1-antibody cDNA containing cassettes is by homologous recombination replacement.